

*B24*  
(SEQ ID NO: 53) and (v) SKDGKKKKKSCTK (SEQ ID NO: 54), wherein the N-terminus is on the left,

and

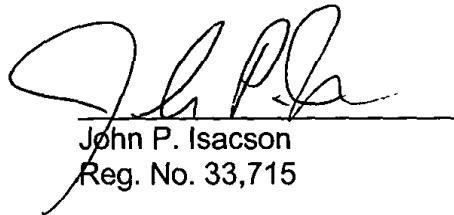
(B) a fatty acid N-acyl group of 8 to 18 methylene units at the N-terminus or at an  $\epsilon$ -amino group of a lysine residue of the peptide.

**REMARKS**

Applicants submit this Amendment to insert the required SEQ ID NOS of the Sequence Listing filed concurrently herewith and to indicate the insertion point for the Sequence Listing.

Applicants respectfully request examination on the merits of this application.

Respectfully submitted,



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Date

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**Amendments to specification with brackets and underlining**

**Page 6, paragraph 3, line 23:** Please amend as follows:

Suitable examples of amino acid sequences comprising basic amino acids include:

- (i) DGPKKKKKKSPSKSSG (SEQ ID NO: 50)
- (ii) GSSKSPSKKKKKPGD (SEQ ID NO: 51)
- (iii) SPSNETPKKKKKRFSFKKSG (SEQ ID NO: 52)
- (iv) DGPKKKKKKSPSKSSK (SEQ ID NO: 53)
- (v) SKDGKKKKKKSKTK (SEQ ID NO: 54)

(N-terminus on left)

**Page 6, paragraph 4, line 31:** Please amend as follows:

Examples of amino acid sequences derived from ligands of known integral membrane proteins include RGD-containing peptides such as GRGDSP (SEQ ID NO: 55) which are ligands for the  $\alpha_{\text{IIb}}\beta_3$  integrin of human platelet membranes. Another example is DGPSEILRGDFSS (SEQ ID NO: 56) derived from human fibrinogen alpha chain, which binds to the GpIIb/IIIa membrane protein in platelets.

**Page 6, paragraph 5, line 36:** Please amend as follows:

Further examples of such sequences include those known to be involved in interactions between membrane proteins such as receptors and the major histocompatibility complex. An example of such a membrane protein ligand is the sequence GNEQSFRVDLRTLRYA (SEQ ID NO: 57) which has been shown to bind to the major histocompatibility complex class 1 protein (MHC-1) with moderate affinity (L. Olsson *et al.*, Proc. Nat. Acad. Sci. USA. 91, 9086-909, 1994).

**Page 7, paragraph 2, line 5:** Please amend as follows:

Yet further examples of such sequences employ a membrane insertive address specific for T-cells. Such sequence is derived from the known interaction of the transmembrane helix of the T-cell antigen receptor with CD3 (Nature Medicine 3, 84-88, 1997). Examples are peptides containing the sequence GFRILLKV (SEQ ID NO: 58) such as:

SAAPSSGFRILLKV (SEQ ID NO: 59)

AAPSVIGFRILLKVAG (SEQ ID NO: 60)

**Page 22, paragraph 5, line 19:** Please amend as follows:

The soluble CR1 polypeptide is derivatised in accordance with the invention by any convenient strategy from those outlined above. In a preferred embodiment the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from

DGPKKKKKSPSKSSGC (SEQ ID NO: 61)

GSSKSPSKKKKKPGDC (SEQ ID NO: 5)

CDGPKKKKKSPSKSSK (SEQ ID NO: 18)

SKDGKKKKKSCTKC (SEQ ID NO: 19)

CSAAPSSGFRILLKV (SEQ ID NO: 20)

AAPSVIGFRILLKVAGC (SEQ ID NO: 62)

and

DGPSEILRGDFSSC (SEQ ID NO: 63)

(N-terminus on left).

**Page 36, paragraph 1, line 1:** Please amend as follows:

(a) Construction of plasmid pDB1031 encoding SCR1-3/switch

Fragment 1 and fragment 2 of pDB1031-5 were the same as Example 6 above. Two oligonucleotides, SEQ ID No. 3 and SEQ ID No. 4, prepared by Cruachem were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has an *Ban*I/*Eco*RI overhang and duplicates the sequence at the 3' end of pDB1031-5 but

in addition contains 17 additional codons coding for DGPKKKKKSPSKSSGC (SEQ ID NO: 61) just before the stop codon. This is fragment 4.

**Page 36, paragraph 2, line 8:** Please amend as follows:

Fragments 1, 2 and 4 were ligated with T4 DNA ligase in a single reaction to give pDB1031. The ligated plasmid was transformed into competent *E. coli* JM109. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR1-3 (SEQ ID 27 of WO 94/00571) had been altered by C terminal addition of amino acids DGPKKKKKSPSKSSGC (SEQ ID NO: 61) to give SEQ ID NO: 7.

**Page 41, paragraph 2, line 17:** Please amend as follows:

A suitable example of a modified terminus of the cDNA sequence of sCR1 is as follows:

(5909)      Bal I (5914)

.....CCT CTG GCC AAA TGT ACC TCT CGT GCA CAT TGC TGA (SEQ ID NO: 64)

The codon Asp-1930 in CR1 is replaced by that for a Cysteine (followed by a stop codon to generate a soluble protein) through ligation of a modified oligonucleotide to the unique Bal I restriction endonuclease site at position 5914 (numbering from Fearon et al, 1989, 1991).

**Page 46, paragraph 5, line 12:** Please amend as follows:

(a) Construction of plasmid pBC04-29 encoding [SCR1-3(deIN195-K196)TANANKSLSSISCQT (SEQ ID NO: 14)

**Page 46, paragraph 6, line 14:** Please amend as follows:

Plasmid pBC04-29 was constructed from plasmid pDB1013-5 encoding SCR1-3 of LHR-A of CR1 (patent application WO 94/00571) by QuickChange site directed

mutagenesis (Stratagene) according to the manufacturers protocols. Two complementary oligonucleotides (SEQ ID No 15 and SEQ ID No 16) were used to generate a novel restriction site (silent) at G186/P187 and a C terminal cysteine. In the event the reaction produced a frame-shift mutation at position N195. In the resulting sequence the C terminal amino acids N195 and K196 are replaced with a 14 amino acid peptide TANANKSLSSISCQT (SEQ ID NO: 14). Fortuitously, this sequence contains an internal cysteine close to the C terminus, preceded by a spacer sequence of 11 amino acids.

**Page 46, paragraph 7, line 24:** Please amend as follows:

(b) Expression of plasmid pBC04-29 encoding [SCR1-3(delN195-K196)TANANKSLSSISCQT (SEQ ID NO: 14) in *E. coli*

**Page 47, paragraph 1, line 1:** Please amend as follows:

(c) Isolation and purification of [SCR1-3(delN195-K196)TANANKSLSSISCQT (SEQ ID NO: 14)

**Page 47, paragraph 3, line 30:** Please amend as follows:

[SCR1-3(delN195-K196)TANANKSLSSISCQT (SEQ ID NO: 14) prepared as described in Example 19 (approx. 30uM protein; 0.1ml) was mixed with TCEP (5nM in 50mM Hepes pH 4.5; 0.0072ml) and incubated at room temperature (22 degrees C) for 15h. 0.05ml of this solution was mixed with 0.005ml of 0.5M ethanolamine and 0.003ml of 7mM MSWP-1 (see Example 2); the solution was incubated for a further 4h at room temperature. SDS PAGE analysis showed a major band in the preparation had an apparent molecular weight of 25 000, clearly shifted from the original parent molecular weight of 23 000.

**Page 48, paragraph 1, line 1:** Please amend as follows:

Example 21 Preparation of [SCR1-3]DGPSEILRGDFSSC (SEQ ID No. 23)

(a) Construction of plasmid pBC04-31 encoding [SCR1-3]DGPSEILRGDFSSC  
(SEQ ID NO: 23)

Plasmid pBC04-31 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 25 and SEQ ID No. 26). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to  $[>90^{\circ}\text{C}]$   $>90^{\circ}\text{C}$  and slowly cooling to room temperature and ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for the presence of a novel Aval site at position 2733. On digestion with Aval pBC04-31 yielded fragments of 2311 and 495bp. DNA from positive clones was used to transform the expression strains. The oligonucleotides inserted added the peptide sequence DGPSEILRGDFSSC (SEQ ID NO: 23) to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

**Page 48, paragraph 2, line 16:** Please amend as follows:

(b) Expression, isolation and purification of [SCR1-3]DGPSEILRGDFSSC (SEQ ID NO: 23)

Expression, isolation and purification of [SCR1-3]DGPSEILRGDFSSC (SEQ ID NO: 23) is carried out using pBC04-31 by procedures generally described in Example 6.

**Page 48, paragraph 3, line 31:** Please amend as follows:

[SCR1-3]DGPSEILRGDFSSC (SEQ ID NO: 23) protein prepared in a similar way to that described in Example 21 is reacted with MSWP-1 as described in Example 8 to give the title compound.

**Page 48, paragraph 4, line 35:** Please amend as follows:

Example 23 Preparation of [SCR1-3]AAPSVIGFRILLKVAGC (SEQ ID No. 33)

(a) Construction of plasmid pBC04-34 encoding [SCR1-3] AAPSVIGFRILLKVAGC (SEQ ID NO: 33)

**Pag 49, paragraph 1, line 1:** Please amend as follows:

Plasmid pBC04-34 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 34 and SEQ ID No. 35). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to >90°C and slowly cooling to room temperature and were ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for an increase in size of the NdeI/HindIII fragment by 59 base pairs. The presence of the cysteine codon was determined by the presence of a Ddel site at position 2781. pBC04-34 digested with Ddel yielded diagnostic bands of 481 and 109bp. DNA from positive clones was used to transform the expression strains (see next section). The oligonucleotides inserted added the peptide sequence AAPSVIGFRILLKVAGC (SEQ ID NO: 62) to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

**Page 49, paragraph 2, line 15:** Please amend as follows:

(b) Expression, isolation and purification of [SCR1-3]APSVIGFRILLKVAGC (SEQ ID NO: 36)

Expression, isolation and purification of [SCR1-3]APSVIGFRILLKVAGC (SEQ ID NO: 36) is carried out using pBC04-34 by procedures generally described in Example 6.

**Page 49, paragraph 3, line 29:** Please amend as follows:

[SCR1-3]AAPSVIGFRILLKVAGC (SEQ ID NO: 33) protein prepared in a similar way to that described in Example 23 is reacted with MSWP-1 as described in Example 8.

**Page 59, paragraph 2, line 7:** Please amend as follows:

**pET15b vector for DAF xpr ssion**

The pET15b expression vector is a T7 promotor based vector available commercially through Novagen (Wisconsin, USA). Briefly, the vector carries the following features which make it a useful vehicle for the expression of heterologous proteins in *E. coli*; a selectable antibiotic marker ( $\beta$ -lactamase) conferring ampicillin resistance, a copy of the *lacI* gene providing *lac* repression in strains of *E. coli* that are *lacI*<sup>r</sup>, and the T7-*lac* promoter. The T7-*lac* promoter combines the T7 RNA polymerase promoter sequences with the *lacI* repressor binding site from the *E. coli* lactose operon. This reduces expression of the cloned gene in the absence of the inducer isopropyl  $\beta$ -D thiogalactopyranoside (IPTG). Downstream of the T7 promoter is a multiple cloning site built into a region of sequence which codes for a polyhistidine tag sequence.

Translation initiates at the methionine codon at position 332-330 of the vector sequence and proceeds counter-clockwise to yield the following peptide:

MGSSHHHHHSSGLVPRGSH (SEQ ID NO: 65). The six histidine residues allow for purification of the fusion protein by metal chelation chromatography, whilst the GLPVR (amino acids 13-17 of SEQ ID NO: 65) motif constitutes a thrombin cleavage site for removal of the peptide from the fusion protein after purification. Three restriction enzyme sites are provided for the insertion of cloned DNA in-frame with the polyhistidine leader. These are *Nde*I (CATATG), *Xba*I (CTCGAG) and *Bam*HI (GGATCC). Use of the *Nde*I site to overlap the methionine initiation codon of the cloned gene removes the possibility of unwanted amino acids at the N-terminus of the fusion protein. At the 3' end of the multiple cloning site is the T7 transcriptional terminator.

**Page 70, paragraph 3, line 15:** Please amend as follows:

**Example 33: A method for the synthesis and characterization of APT2057 (SEQ ID NO: 46)**

APT2057 is a protein that comprises the short consensus repeats 2,3 and 4 of human CD55 (decay accelerating factor, DAF), with a carboxyl terminal cysteine residue and

an amino terminal histidine tag motif expressed in a recombinant form in *E. coli* cells. cDNA to human DAF mRNA was generated from total brain RNA (OriGene Technologies, USA). Reverse transcription was primed with 40 pmol of primer DAF-R (5'GGAATTCTAAGTCAGCAAGCCATGGTTACT 3') (SEQ ID NO: 66), 3 µg human brain total RNA and other reagents as recommended by the the RT system manufacturers (Promega, Southampton, UK). Half of the RT reaction (10 µl) was used as template for PCR. Reaction volume was increased to 50 µl by the addition of water, buffer, MgCl<sub>2</sub> (to 2 mM), DMSO (to 5%) and 20 pmol oligonucleotide DAF-F (5'GCATATGACCGTCGCGCGGCCGAGC 3') (SEQ ID NO: 67). One unit of Taq polymerase (MBI Fermentas, Vilnius, Lithuania) was added, and the reaction subjected to 35 cycles of PCR (94°C, 30 sec; 64°C, 30 sec; 72°C, 60 sec). A PCR product of 1156 bp was identified by agarose gel electrophoresis, purified from the gel and ligated using standard procedures into the T-cloning vector pUC57/T (MBI-Fermentas, Vilnius, Lithuania). Positive clones were identified by PCR screen, analysed by plasmid restriction map and confirmed by full sequence analysis. A plasmid to encode APT2057 was generated by PCR using the pUC-DAF plasmid as template. Primers were designed to amplify the region of the DAF gene encoding amino acids 97-285 (SCR2-4). The 5' primer incorporated an NdeI restriction enzyme site, and a codon specifying glutamine, thereby introducing an amino terminal methionine-glutamine amino acid pair. The 3' primer added a carboxyl terminal cysteine residue and incorporated an EcoRI restriction enzyme site. The PCR product was cloned into the pUC57/T T-vector as described, sequenced, the insert excised with NdeI and EcoRI, and ligated into pET15b (Novagen, Madison, USA, see Methods section). The product of this ligation is the plasmid pET100-02, which expresses DAF(SCR2-4) as an in-frame fusion of a 20 amino acid leader sequence (MGSSHHHHHSSGLVPRGSH) (SEQ ID NO: 65) to the 191 amino acid DAF SCRs2-4. pET100-02 DNA was introduced into *E. coli* HAMS113 and transformed cells selected by virtue of their ability to grow on LB+agar plates in the presence of 50 µg/ml ampicillin (LBAMP). A single colony representing HAMS113 containing DNA with the coding capacity for APT2057 was grown overnight at 37°C with shaking (200 rpm) in LBAMP medium, then diluted 1:100 into 1 litre fresh LBAMP and growth at 37°C with shaking. Growth was monitored by measurement of culture

turbidity at 600nm, and upon reaching an optical density of 0.6, isopropyl  $\beta$ -D thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, followed by a further 3 hours of growth under the same conditions as described above. The expression of APT2057 was analysed by SDS-PAGE (described in methods). APT2057 appeared as a unique protein product of approximately 24000 Da as estimated by comparative mobility with molecular weight standards. Cells containing APT2057 are harvested by centrifugation and inclusion bodies isolated as follows. Briefly, the cells are resuspended in lysis buffer (50 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA), 50 mM NaCl, pH 8.0) at 50 ml per litre of initial culture. The suspension is lysed by two passages through an Emulsiflex homogeniser (Glen-Creston, Middlesex UK), followed by centrifugation at 15000 x g to purify inclusion bodies. Inclusion bodies are initially resuspended to approximately 1 mg.ml<sup>-1</sup> (as estimated from SDS-PAGE) in 20 mM Tris, 1 mM EDTA, 50mM 2-mercaptoethanol, pH8.5, and subsequently diluted to a final concentration of 8M urea by the addition of 10 M urea 20 mM Tris, 1 mM EDTA, 50mM 2-mercaptoethanol, pH8.5. This suspension is stirred at 4°C for 16 hours, and insoluble material removed by centrifugation at 15000 x g for 30 minutes. The APT2057 is refolded by 1 in 50 dilution into 20 mM ethanolamine, 1 mM EDTA, pH 11 buffer and static incubation at 4°C for 24 hours. Insoluble material is removed by centrifugation (10000 x g, 10 minutes), and soluble material buffer exchanged into Dulbecco's A PBS, pH 7.4 using an XK50 x 23 cm Sephadex G25 column. Refolded APT2058 is analysed by SDS-PAGE, Western blot and the effectiveness of the protein in a haemolytic assay (described in methods).

**Page 72, paragraph 2, line 12:** Please amend as follows:

**Example 34: A method for the synthesis and characterization of APT2058 (SEQ ID NO: 47)**

APT2058 is a protein that comprises the short consensus repeats 1,2,3 and 4 of human CD55 (decay accelerating factor, DAF), with a carboxyl terminal cysteine residue and an amino terminal histidine tag motif expressed in a recombinant form in *E. coli* cells. cDNA to human DAF mRNA was generated from total brain RNA as described in

Example 9. A plasmid to encode APT2058 was generated by PCR using the pUC-DAF plasmid as template. Primers were designed to amplify the region of the DAF gene encoding amino acids 35-285 (SCR1-4). The 5' primer incorporated an NdeI restriction enzyme site, and a codon specifying glutamine, thereby introducing an amino terminal methionine-glutamine amino acid pair. The 3' primer added a carboxyl terminal cysteine residue and incorporated an EcoRI restriction enzyme site. The PCR product was cloned into the pUC57/T T-vector as described, sequenced, the insert excised with NdeI and EcoRI, and ligated into pET15b (Novagen, Madison, USA). The product of this ligation is the plasmid pET99-02, which expresses DAF (SCR1-4) as an in-frame fusion of a 20 amino acid leader sequence (MGSSHHHHHSSGLVPRGSH) (SEQ ID NO: 65) to the 251 amino acid DAF SCR1-4 (APT2058). pET99-02 DNA was introduced into *E. coli* HAMS113 (see methods) and expression of the recombinant protein induced as described in Example 1. The expression of APT2058 was analysed by SDS-PAGE (described in methods). APT2058 appeared as a unique protein product of approximately 31000 Da as estimated by comparative mobility with molecular weight standards. Cells containing APT2058 were harvested by centrifugation and inclusion bodies isolated as follows. Briefly, the cells were resuspended in lysis buffer (50 mM Tris, 1 mM ethylene diamine tetra-acetic acid (ETDA), 50 mM NaCl, pH 8.0) at 50 ml per litre of initial culture. The suspension was lysed by two passages through an Emulsiflex homogeniser (Glen-Creston, Middlesex UK), followed by centrifugation at 15000 x g to purify inclusion bodies. Inclusion bodies were initially resuspended to approximately 1 mg.ml<sup>-1</sup> (as estimated from SDS-PAGE) in 20 mM Tris, 1 mM EDTA, 50mM 2-mercaptoethanol, pH8.5, and subsequently diluted to a final concentration of 8M urea by the addition of 10 M urea 20 mM Tris, 1 mM EDTA, 50mM 2-mercaptoethanol, pH8.5. This suspension was stirred at 4°C for 16 hours, and insoluble material removed by centrifugation at 15000 x g for 30 minutes. The APT2057 was refolded by 1 in 50 dilution into 20 mM ethanolamine, 1 mM EDTA, pH 11 buffer and static incubation at 4°C for 24 hours. Insoluble material was removed by centrifugation (10000 x g, 10 minutes), and soluble material buffer exchanged into Dulbecco's A PBS, pH 7.4 using an XK50 x 23 cm Sephadex G25 column. Refolded APT2058 was analysed by SDS-PAGE, Western blot and the effectiveness of the protein in a

haemolytic assay (described in methods). Using this assay (at 1:400 dilution of human serum), the concentration of APT2058 required to bring about 50 % inhibition of lysis ( $IC_{50}$ ) was approximately 3 nM.

**IN THE CLAIMS:**

Please amend the claims as follows:

11. (Amended) A derivative according to claim 10 wherein the amino acid sequence is selected from:

- i) DGPKKKKKSPSKSSG (SEQ ID NO: 50)
- ii) GSSKSPSKKKKKPGD (SEQ ID NO: 51)
- iii) SPSNETPKKKKKRFSFKKSG (SEQ ID NO: 52)
- iv) DGPKKKKKSPSKSSK (SEQ ID NO: 53)
- v) SKDGKKKKKSCTK (SEQ ID NO: 54)

(N-terminus on left)

12. (Twice Amended) A derivative according to claim 8 wherein a membrane binding element is a ligand of a known integral membrane protein selected from GRGDSP (SEQ ID NO: 55), DGPSEILRGDFSS (SEQ ID NO: 56), GNEQSFRVDLRTLRYA (SEQ ID NO: 57), GFRILLKV (SEQ ID NO: 58), SAAPSSGFRILLKV (SEQ ID NO: 59) and AAPSVIGFRILLKVAG (SEQ ID NO: 60) or the carbohydrate ligand Sialyl Lewis\*.

21. (Amended) A soluble derivative according to claim 20 wherein the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from

- DGPKKKKKSPSKSSGC (SEQ ID NO: 61)
- GSSKSPSKKKKKPGDC (SEQ ID NO: 5)
- CDGPKKKKKSPSKSSK (SEQ ID NO: 18)
- SKDGKKKKKSCTKC (SEQ ID NO: 19)

CSAAPSSGFRILLLKV (SEQ ID NO: 20)

AAPSVIGFRILLLKVAGC (SEQ ID NO: 62)

and

DGPSEILRGDFSSC (SEQ ID NO: 63)

(N-terminus on left).

52. (Amended) A peptidic membrane binding element derivatised according to claim 32, wherein the peptide has

(A) an amino acid sequence selected from the group consisting of (i)

DGPKKKKKSPSKSSG (SEQ ID NO: 50), (ii) GSSKSPSKKKKKPGD (SEQ ID NO:

51), (iii) SPSNETPKKKKKRFSFKKSG (SEQ ID NO: 52), (iv) DGPKKKKKKSPSKSSK

(SEQ ID NO: 53) and (v) SKDGKKKKKS GTK (SEQ ID NO: 54), wherein the N-

terminus is on the left,

and

(B) a fatty acid N-acyl group of 8 to 18 methylene units at the N-terminus or at an  $\epsilon$ -amino group of a lysine residue of the peptide.